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INTRACEREBRAL BISPECIFIC LIGAND-ANTIBODY CONJUGATE INCREASES SURVIVAL OF ANIMALS BEARING ENDOGENOUSLY ARISING BRAIN TUMORS

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Bispecific antibodies capable of simultaneously binding a tumor surface antigen and the T-cell receptor/CD3 complex are capable of inducing polyclonal immune effector cells to destroy targeted tumor cells. Bispecific antibody immunotherapies have shown some promise against tumors of hematopoietic origin such as lymphomas, but use of bispecific antibodies for the treatment of solid tumors has been less fully explored. To test the preclinical potential of bispecific antibody therapy against an endogenously arising solid brain tumor, we have utilized a novel variation of conventional bispecific antibodies, referred to as bispecific ligand-antibody conjugates, to target choroid plexus tumors. The bispecific ligand-antibody conjugate described in this study is a chemical conjugate between an anti-CD3 monoclonal antibody (MAb) and folic acid, the ligand for a high-affinity surface receptor expressed on the surface of choroid plexus tumors. SV11 mice transgenic for SV40 large T antigen and its promoter develop solid choroid plexus tumors in the brain. We demonstrate that choroid plexus tumor cells are susceptible *in vitro* to cytolysis mediated by cytotoxic T cells in the presence of the bispecific ligand-antibody conjugate in a folate-inhibitable manner. Adoptive immunotherapy studies demonstrate the potential benefits of the bispecific ligand-antibody conjugate *in vivo*. The bispecific conjugate is capable of retaining adoptively transferred T lymphocytes specifically within tumor tissue for periods of up to at least 1 week. Further, following intracerebro-ventricular injection of bispecific conjugate and splenocytes containing activated cytotoxic T cells, T cells were observed to penetrate to interior regions of the tumor. A single treatment of adoptively delivered activated effectors and bispecific conjugate into the brain ventricles was insufficient to produce significant increases in survival of SV11 mice, but repeated treatment through indwelling cannulas prolonged survival of animals treated with activated effectors and bispecific ligand-antibody conjugate compared to animals treated with activated effectors or saline alone. Our results demonstrate that the SV11 model may be useful for preclinical evaluation and optimization of bispecific ligand-antibody conjugate treatments of solid tumors. *Int. J. Cancer* 78:470–479, 1998.

Tumors may evade immune recognition via a number of mechanisms. The lack of appropriate antigen presentation and/or costimulation by tumors is one means of preventing specific T-lymphocyte recognition. In addition, the microenvironment in which a tumor arises may actively suppress the initiation or execution of a competent specific immune response to tumor.

The brain has classically been considered an immune privileged site. The blood-brain barrier (BBB) is thought to prevent trafficking of unactivated lymphocytes through brain parenchyma, and the decreased surveillance may lower the probability of specific recognition of brain tumors. Molecular factors blocking the activation of immune effectors cells [*e.g.,* transforming growth factor-β (TGF-β) or prostaglandin E_2] are released by cells in the brain including microglia, astrocytes and certain types of brain tumors (Fabry *et al.,* 1995). Systemic release of immunosuppressive factors by some brain tumors may further hamper the ability of the immune system to mount or maintain a response to tumor and, in some cases, may cause systemic immunosuppression (Van Meir, 1995). In light of these concerns, therapeutic strategies that overcome endogenous lymphocyte activation barriers may be useful.

One means of circumventing possible endogenous activation barriers is to activate effector lymphocytes *ex vivo.* Some clinical studies have focused on adoptive transfer of lymphokine activated killer (LAK) cells (Hayes *et al.,* 1995; Smith *et al.,* 1996). Some patients showed improvement when treated with LAK cells, but the recurrence of primary malignancy was high. This finding may be due to the polyclonal nature of the LAK cells that largely are not specific to the tumor under treatment. Adoptive transfer studies may benefit from approaches that redirect polyclonal effector cells to specifically lyse tumor cells.

A strategy that has been shown to redirect cytolytic action of polyclonal cytotoxic T cells (CTL) specifically against various tumors is bispecific antibodies. Simultaneously specific for a tumor surface antigen and an immune effector triggering molecule, bispecific antibodies can juxtapose CTL to tumor cells and mediate the specific redirection of polyclonal immune effectors against tumor cells. Bispecific antibodies circumvent the requirement for tumor antigen presentation to CTLs by directly signaling via immune effector surface molecules (*e.g.,* T-cell receptor/CD3 complex) responsible for stimulating cytotoxic action. Variations of the bispecific antibody strategy have been shown to be successful in numerous animal models and are currently being employed in clinical trials (*e.g.,* Canevari *et al.,* 1995).

Whereas most animal studies employing bispecific antibodies have concentrated on hematopoietic tumors, solid tumors may be a more difficult challenge. Targeting and maintaining sufficient immunotherapeutic reagents or active immune effector cells in the interior of tumors may be an obstacle to bispecific antibody treatment of solid tumors. As with some other immunotherapeutic strategies (*e.g.,* transfection of tumor cells with immune activating cytokines), reported success against solid tumors using bispecific antibody therapy is generally limited to preventing the growth of small tumor burdens transplanted into animal models rather than rejecting an established, solid tumor of endogenous origin.

One model for endogenously arising brain tumors is SV11 mice that are transgenic for large T antigen (Tag) and its associated promoter derived from the simian virus 40 (SV40) genome (Van Dyke *et al.,* 1985, 1987). SV11 mice develop choroid plexus tumors (CPT) with 100% penetrance and become moribund at approximately 100 days of age (Van Dyke *et al.,* 1987). The mechanism of tumorigenesis in SV11 mice involves direct inhibition of tumor suppressors p53 and pRB by Tag in choroid plexus epithelial cells. The reason for the selective expression of Tag in the choroid plexus epithelium is unknown. SV40 Tag sequences have been found in a majority of pediatric ependymomas and half of the

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pediatric CPTs tested (Bergsagel *et al.*, 1992). In fact, intact virions of SV40 have been isolated from some of these tumors (Lednicky *et al.*, 1995). Speculation of a viral etiology for pediatric ependymomas and CPTs suggests that CPTs which develop in SV11 mice may be analogous to the development of their human counterparts and hence useful for the study of various immunotherapeutic regimes aimed at eliminating solid tumors.

Previously, we described a novel bispecific antibody targeting strategy where the small ligand folic acid was chemically coupled to anti-T-cell receptor (TCR) antibodies (Kranz *et al.*, 1995). These bispecific ligand-antibody conjugates demonstrated specific and sensitive ability to redirect CTLs to lyse tumor cells expressing the high-affinity folate receptor (FR) *in vitro*. FR has been suggested as a potential tumor-associated antigen useful for immunotherapeutic targeting of certain human neoplasms (Bolhuis *et al.*, 1992). Among neoplasms expressing FR are CPT, ependymomas, >95% of ovarian carcinomas and approximately 30% of mammary adenocarcinomas (Buist *et al.*, 1993; Coney *et al.*, 1991; Ross *et al.*, 1994). SV11 CPTs express FR and thus may be useful for testing and optimizing the bispecific ligand-antibody conjugate strategy *in vivo* (Patrick *et al.*, 1997). We have shown that treatment of SV11 mice with a single-chain Fv antibody against Vβ8 TCRs conjugated with folate caused T-cell infiltration of the tumors and significantly prolonged survival (Roy *et al.*, 1998). In the present study, we used an anti-CD3 antibody conjugated to folic acid that targets all T cells, and delivered the bispecific ligand-antibody conjugate directly into the brain.

We first determined whether an anti-CD3 antibody/folate conjugate is capable of redirecting CTL against CPT cells. The anti-murine CD3 antibody 2C11 conjugated to folate was capable of redirecting both monoclonal and polyclonal CTls to specifically lyse CPT cells *in vitro*. Adoptive transfer of polyclonal CTL and 2C11/folate conjugate directly in the lateral ventricles of the brain of SV11 animals led to retention of T lymphocytes in CPT within the interior of the tumor. In contrast, rapid clearance of CTL from the brain was observed in the absence of bispecific ligand-antibody conjugate. Finally, continued adoptive therapy of SV11 mice with CTL and bispecific ligand-antibody conjugate conferred a significant improvement in survival compared to animals treated with CTL or saline alone.

MATERIAL AND METHODS

Mice

C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed in animal facilities at the University of Illinois. The SV11 transgenic line was obtained from T.A. Van Dyke (University of North Carolina). SV11 males heterozygous for Tag were mated to C57BL/6 females. Progeny were screened by polymerase chain reaction (PCR) for the presence of Tag (Patrick *et al.*, 1997). Prior to use in adoptive transfer treatment regimes, mice were placed on low-folate chow for a period of at least 1 week to reduce the level of serum folates that could potentially compete with the bispecific ligand-antibody conjugate for binding to FRs on CPT. All studies described were approved by the Laboratory Animal Care Advisory Committee and conducted in accordance with NIH guidelines for the care and handling of laboratory animals in experimental studies.

Cell lines and antibodies

All cell lines were incubated in a humidified incubator at 37°C and 5% CO_2. F2-MTX^{r}A, a non-adherent DBA/2-derived erythroleukemia line (Brigle *et al.*, 1991), was maintained in RPMI 1640 medium containing 5 mM HEPES, 10% (vol/vol) heat-inactivated fetal bovine serum, 1.3 mM L-glutamine, 50 mM 2-mercaptoethanol, 100 units/ml penicillin and 100 units/ml streptomycin. Cytotoxic T-lymphocyte clone 2C, a mouse alloreactive cell line specific for L^d, was maintained in the same RPMI medium described above and supplemented with 10% (vol/vol) supernatant from concanavalin A-stimulated rat spleen cells, 5% α-methylmannoside and mitomycin C-treated BALB/c spleen cells (L^d) as stimulators (Kranz *et al.*, 1984). Hybridoma 2C11 (Leo *et al.*, 1987), a hamster IgG specific for the murine CD3ϵ subunit, was purified from ascites by ammonium sulfate precipitation and protein A-Sepharose. Hybridoma 37.51 (Gross *et al.*,1992), a hamster IgG-secreting line specific for murine CD28, was cultured in serum-free RPMI 1640 medium and purified by passage over a protein G-Sepharose column. Rabbit antiserum to murine FR (kindly provided by Dr. K. Brigle) and normal rabbit antiserum (kindly provided by S. Miklasz) were utilized in flow cytometry without further processing.

Flow cytometry

CPT cells were mechanically dissociated through wire mesh and separated by sedimentation into individual cells and small aggregates. Red blood cells were lysed by incubation in lysing buffer (0.14 M NH_4Cl, 0.017 M Tris, pH 7.2) for 5 min at 37°C. Isolated CPT cells were incubated with polyclonal rabbit antiserum to murine FR followed by fluorescent goat anti-rabbit secondary (Kirkegaard and Perry, Gaithersburg, MD) or secondary antibody alone. Cells were analyzed on a Modified Coulter EPICS 753 flow cytometer (Coulter, Miami, FL) with Cyclops version 3.14 software for the percentage of CPT cells expressing FR. Splenocytes were labeled with a combination of fluorescent anti-CD4, CD8 and CD69 primary antibodies (Pharmingen, San Diego, CA). Cells were analyzed on a Coulter XL-MCL flow cytometer with Coulter System II software. Dead cells were excluded on the basis of high- or low-angle light scatter.

Preparation of antibody/folate conjugate

Conjugation of folic acid to purified 2C11 was performed as described previously (Kranz *et al.*, 1995). Briefly, folic acid (Sigma, St. Louis, MO) was dissolved in dimethylsulfoxide (DMSO) to a final concentration of 6.7 mM. EDC [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; Pierce, Rockford, IL] was added to a final concentration of 33.5 mM (1:5 folate/EDC ratio) and incubated for 30 min at room temperature in the dark. Reacted folate/EDC was added to 2C11 antibody (1 mg/ml) in 0.1 M MOPS, pH 7.5, at a 100:1 folate/antibody ratio. Further incubation for 1 hr at room temperature in the dark was followed by passage over a Sephadex G-25 (Sigma) column equilibrated in phosphate-buffered saline (PBS). The excluded-peak fractions containing conjugated antibody were pooled and analyzed spectrophotometrically for determination of folate density. Densities averaged 4–8 folates/antibody. 2C11/folate bispectific ligand-antibody conjugates were stored at 4°C in the dark.

Cytotoxicity assays

Mechanically dissociated CPT cells or F2-MTX^r A cells were labeled with 50 μl of ^{51}Cr (2.5 mCi/ml) for 1 hr at 37°C, washed repeatedly in folate-free RPMI 1640 containing 5% (vol/vol) fetal bovine serum and subsequently used in 96-well plate cytotoxicity assays at 2×10^4 cells/well. 2C11/folate conjugate was added to triplicate wells at 150 ng/ml. For folate-inhibition studies, free folate was added at various final concentrations (2.5×10^{-7} to 2.5×10^{-11} M). Activation of CTL clone 2C effector cells was as described above and added at an effector to target ratio of 10:1. Activation of splenocytes from C57BL/6 or SV11 mice was induced by an intravenous (i.v.) injection of 10 μg 2C11 antibody. The spleen was removed from these mice 48 hr later (optimal time for CTL activation, data not shown). Splenocytes were isolated by disruption through wire mesh and red blood cells were lysed by incubation in lysing buffer for 5 min at 37°C. Remaining splenocytes were washed 3 times in folate-free RPMI 1640 medium containing 5% (vol/vol) heat-inactivated fetal bovine serum and added at effector to target ratios of 100:1, 31:1, 10:1, 3:1 or 1:1 as indicated. Plates were incubated at 37°C for 4 hr in a humidified 5% CO_2 incubator. Culture supernatants were removed for γ counting. Specific ^{51}Cr-release was calculated by standard methods [% specific ^{51}Cr-release = (experimental counts − spontaneous counts)/[maximal counts − spontaneous counts) × 100].

In vivo *activation of splenocytes for adoptive transfer*

C57BL/6 mice were injected i.v. with 10 μg anti-CD3 antibody 2C11 and harvested as described under cytotoxicity assays. Splenocytes were washed with PBS rather than folate-free RPMI 1640. In cases were splenocytes and bispecific ligand-antibody conjugate were simultaneously transferred, splenocytes and the conjugate were incubated on ice for at least 30 min.

T-lymphocyte retention in CPT in vivo

SV11 mice aged 90–100 days and having been placed on low-folate chow for a period of not less than 1 week were anesthetized with 1.7 mg/20 g body weight ketamine and 250 μg/20 g body weight xylazine in sterile PBS injected intraperitoneally (i.p.). Mice scalps were shaved and prescrubbed with betadine. Surgery was performed using aseptic techniques. An incision of approximately 1 cm was made along the mid-sagittal plane of the scalp. A stereotaxic device was employed to determine coordinates 1 mm caudal and 1 mm lateral of bregma. The skull was perforated with a drill. Intracerebroventricular (ICV) injections (either saline alone, 2×10^6 activated splenocytes, or 2×10^6 activated splenocytes and 100 ng 2C11/folate conjugate) were delivered 2.5 mm deep of dura (location of superior horn of lateral ventricle) in a total volume of 5–10 μl at a rate of 2 μl/min, using a Hamilton syringe with an attached 28 gauge needle. Following a 5 min latency postdelivery, the syringe was removed and the skull sealed with bone wax. The scalp was sutured or sealed with Vetbond (3M, Minneapolis, MN). Animals were anesthetized with ketamine/xylazine at 24 hr following ICV delivery. Animals were perfused with acetic acid, zinc, formalin (AZF) fixative (Newcomer Supply, Middleton, WI) at room temperature. Brain, spleen and thymus were removed from each animal and bathed in fixative overnight at room temperature.

Tissue preparation and histochemistry

AZF-fixed brains were blocked into 5–6 transverse sections at designated points (mid-IVth ventricle, rostral cerebellum and approximately every 2.5 mm further rostral) the day following fixation and paraffin embedded along with spleen and thymus samples from the same mouse. All brain sections, spleen and thymus to be analyzed by immunohistochemistry were cut and mounted as 3 μm sections on the same slide. Slides were deparaffinized in xylene, rehydrated to PBS, blocked with Superblock (Pierce) and incubated overnight with rabbit anti-human CD3 (murine CD3 cross-reactivity; DAKO, Carpinteria, CA) at 4°C. Primary antibody was followed by biotinylated goat anti-rabbit and subsequently by avidin-biotin-horseradish peroxidase (HRP) (Vector, Burlingame, CA). Nickel-cobalt-enhanced diaminobenzidine (DAB; Pierce) chromagen was used for detection. Slides were counterstained in methyl green, dehydrated and coverslipped with Permount. Sections of spleen and thymus served as both positive and negative controls with respect to regions labeling positive for the presence of CD3. Analysis of tumor area was determined using NIH Image at ×25 magnification. Individual T cells associated with tumor were counted at ×100 magnification for density calculations (T cells/unit area).

Apoptosis tissue stains

Tissue was prepared as above. Adjacent tissue sections were placed on separate slides for comparison of T lymphocyte presence and apoptosis. A TUNEL assay kit (Trevigen, Gaithersburg, MD) was utilized to detect apoptosis. Briefly, tissue was deparaffinized in xylene and an ethanol series followed by several washes in distilled water. Proteinase K (20 μg/ml) was added to each slide, plastic coverslipped and incubated at room temperature for 15 min. Slides were immersed in 2% H_2O_2 in methanol for 5 min before transferring slides to labeling buffer. Manufacturer's labeling reaction mixture was added to each slide, plastic coverslipped and incubated in a 37°C humid chamber for 1 hr. Following labeling reaction, slides were placed in stop buffer for 5 min, washed 3 times with PBS over a 5 min period and incubated with streptavidin-HRP in PBS for 10 min. Slides were washed again in PBS before addition of metal-enhanced DAB chromagen. Following a 5 min incubation, slides were rinsed repeatedly in distilled water and subsequently counterstained with methyl green. Brain parenchyma, thymus and spleen from experimental animals served as apoptosis controls as well as control slides provided by the manufacturer.

Time course of T-lymphocyte retention in CPT in vivo

The protocol for adoptive transfer of splenocytes and 2C11/folate conjugate was repeated exactly as for T lymphocyte retention at 24 hr, but animals were killed at various time points following ICV delivery ranging from 24 hr to 7 days. Four animals receiving activated polyclonal splenocytes and 2C11/folate conjugate were killed at 24 hr, one animal receiving activated polyclonal splenocytes and 2C11/folate conjugate was killed at each successive time point, 3 animals receiving activated polyclonal effectors alone were killed at 24 hr and 3 untreated animals were killed at time zero, as a control for background density of T cells normally found within CPT. Cross-sectional areas of lateral ventricle CPT from at least 4 separate regions of cortex were calculated on NIH Image, and the number of T cells within those areas quantified to yield T-cell density within tumor.

Treatment of SV11 mice with a single adoptive transfer of activated splenocytes

SV11 mice aged 85–90 days, on low-folate chow for at least 1 week, were injected ICV as described above. Mice were distributed to treatment groups randomly with the proportions of each group matched for sex where possible. Mice received either an average of 3.5×10^6 activated splenocytes and 100 ng 2C11/folate (n = 11), 3.5×10^6 activated splenocytes (n = 7) or PBS alone (n = 11). In instances where 2C11/folate was added to effectors for ICV delivery, incubation was at least 30 min on ice prior to ICV delivery. Mice were monitored daily until 1 of 3 criteria establishing morbidity was met, at which time the mouse was killed, perfused, and brain, spleen and thymus were prepared for immunohistochemistry as described above.

Clinical evaluation of mice

Animals were weighed daily and monitored for level of activity and vestibular integrity. Loss of greater than 25% of pretreatment body weight, lack of responsiveness to external stimuli or carrying head at an improper angle or inability to right self were established as sufficient criterion for morbidity classification.

Statistical methods

T-cell densities were compared using the Student's *t*-test. Survival analyses were based on age at first sign of morbidity according to the above criteria. Ages were entered into a standard Kaplan-Meier survival plot. Statistical *p* values were calculated using the log-rank method on JMP software (SAS Institute, Cary, NC). Mean and median ages at morbidity are reported.

Comparison of in vivo- *and* in vitro-*activated splenocytes*

Unactivated splenocytes were harvested from C57BL/6 mice and depleted of red blood cells with lysing buffer as described above. Splenocytes were washed in RPMI 1640 medium containing 10% (vol/vol) heat-inactivated fetal bovine serum. Splenocytes were placed in 24-well tissue culture plates at 1.5×10^6 cells/well with 100 ng/ml anti-CD3 antibody 2C11 and 5 μg/ml to 8 ng/ml anti-CD28 antibody 37.51 or as indicated. Splenocytes were incubated for 72 h at 37°C in a humidified 5% CO_2 incubator. All cell preparations were >95% viable. Splenocytes activated *in vivo* as described above were compared to splenocytes activated *in vitro* by flow cytometry for percent composition of $CD8^+$ cells. Cytotoxic assays similar to those described above using the FR^+ cell line F^2-MTX^rA as tumor target were used to compare killing efficiencies of the 2 T-lymphocyte activation methods. The ER_{50} (effector to target ratio giving half-maximal lysis) was calculated by linear regression of data points in the linear region of the curve

and was used to compare the lytic potential of *in vivo*- and *in vitro*-activated splenocytes.

Treatment of SV11 mice with multiple adoptive transfers of in vitro*-activated splenocytes*

Mice aged 65–70 days were prepared for surgery as described above. Permanent cannulas were placed bilaterally in the skull of SV11 mice. Knotted PE-50 tubing was inserted bilaterally 1.6 mm caudal and 0.6 mm lateral of bregma to a depth of 3 mm below the skull. Cannulas were held in place with dental cement anchored to a "00" machine screw placed approximately 5 mm rostral of the left cannula. In some cases, stylets fashioned from 28 gauge stainless steel tubing were used to prevent cannula occlusion. Animals were allowed a 2 week period to recover from surgery. Morbidity due to surgery was minimal (<5%). At approximately 85 days of age, mice were injected bilaterally (when possible) with 5×10^5 to 2.5×10^6 splenocytes and 100 ng 2C11/folate conjugate (n = 18), 5×10^5 to 2.5×10^6 splenocytes (n = 11) or saline (n = 6) in a total volume of 10 µl. Splenocytes were activated *in vitro* (as described above) with 100 ng/ml 2C11 and 0.5–1 µg 37.51. Splenocytes were harvested and washed 3 times in PBS prior to ICV injection.

RESULTS

FR expression by CPT cells

The first objective was to determine whether CTL could be redirected by the 2C11/folate conjugate to lyse cells from a solid CPT. To establish the proportion of CPT cells that are positive for FR expression and may serve as potential targets for the bispecific ligand-antibody conjugate targeting strategy, a preparation of dissociated CPT cells from an SV11 mouse was labeled with a polyclonal serum to murine high-affinity FR (Fig. 1). Flow cytometric analysis indicated that a large fraction (>97%) of the isolated tumor cells expresses detectable FR.

Susceptibility of CPT cells to monoclonal CTLs in vitro

To test the susceptibility of these CPT cells to CTL-mediated lysis, dissociated CPT cells were incubated with the CTL clone 2C and 2C11/folate conjugate (150 ng/ml) in a standard ^{51}Cr-release assay. Various concentrations of free folate were added to establish if redirection of cytotoxic activity is specifically mediated through FR expressed by the CPT cells. As shown in Figure 2, CTL clone 2C is capable of lysing CPT cells in a folate-dependent manner. CPT cell incubation with CTL clone 2C in the absence of 2C11/folate conjugate resulted in no detectable lysis (data not shown), further suggesting that the 2C11/folate conjugate is responsible for the redirection of 2C toward FR-expressing cells.

Susceptibility of CPT cells to polyclonal CTLs in vitro

Having established that CPT cells were susceptible to CTL clone 2C, we next determined whether activated splenocytes containing an activated population of polyclonal CTLs derived from C57BL/6 or SV11 mice could be redirected to lyse CPT cells *in vitro*. C57BL/6 or SV11 mice were injected i.v. with 10 µg of 2C11 antibody, which produces an active splenocyte subpopulation of CTL capable of cytolysis 24–48 hr later as measured by standard ^{51}Cr-release assays (data not shown). Splenocytes were harvested from these animals 48 hr after 2C11 administration and utilized in a standard ^{51}Cr-release assay against CPT cells. Figure 3*a* demonstrates that in the absence of 2C11/folate conjugate, both activated and unactivated populations of splenocytes have negligible lytic activity toward CPT cells in a 4 hr assay. Figure 3*b* shows that the presence of 2C11/folate conjugate and activated CTL from either C57BL/6 or SV11 mice can be redirected to lyse CPT cells, while CTLs not preactivated are incapable of inducing lysis in a 4 hr assay. These data indicate that CPT cells are susceptible to lysis by activated polyclonal CTLs from either SV11 or C57BL/6 mice and that 2C11/folate conjugate is a capable mediator of redirecting the lytic potential of these CTLs. Similar to the data presented for monoclonal CTL 2C, the redirection of lytic activity by these *in vivo*-activated CTLs was inhibitable by addition of free folate (data not shown).

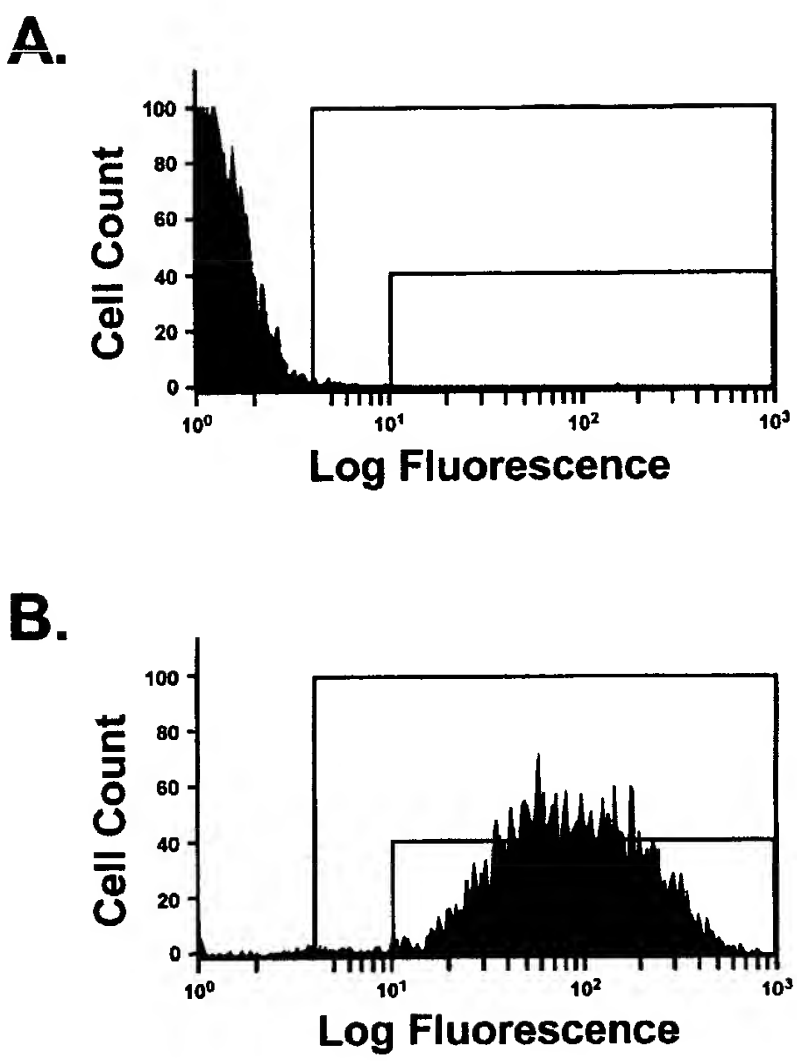


FIGURE 1 – Flow cytometric analysis of high-affinity FR expression. Mechanically dissociated SV11 CPT cells were labeled in the presence of normal rabbit antiserum *(a)* or a rabbit polyclonal serum to murine FR *(b)*. Each was followed by an immunofluorescent goat anti-rabbit secondary antibody.

2C11/folate conjugate retains T cells within CPTs in vivo

To assess the action of the bispecific ligand-antibody conjugate at juxtaposing activated polyclonal CTLs against CPT *in vivo*, we utilized preparations of activated C57BL/6 splenocytes described above incubated with 2C11/folate conjugate or vehicle. An average of 2×10^6 cells suspended in PBS was adoptively transferred directly into the superior lateral ventricles in a volume of 5–10 µl. Animals were killed 24 hr following ICV delivery. Splenocytes injected in the absence of 2C11/folate conjugate showed few T cells associating with CPT after 24 hr (Fig. 4*b*). In contrast, when splenocytes were injected with 2C11/folate conjugate, a significant increase in the density of T cells was present throughout CPT 24 hr later ($p < 0.01$ *vs.* splenocytes without 2C11/folate conjugate) (Fig. 4*a*). Many of the T cells associating with CPT appear to have penetrated to interior depths of the solid tumor. Further, in both 2C11/folate conjugate-treated and untreated animals, few T cells are found in normal brain parenchyma at 24 hr and those observed were typically clustered along myelin tracts or adjacent to vessels, suggesting an outward emigration.

Adjacent tissue sections were analyzed separately for the presence of $CD3^+$ cells or apoptosis 24 hr after ICV administration of activated splenocytes and 2C11/folate conjugate (Fig. 4*c,d*). Apoptotic cells were clustered variously throughout tumor tissue. Juxtaposed to many of the apoptotic cells are $CD3^+$ cells, but the apoptotic cells do not simultaneously stain for the CD3 antigen. Apoptosis was not detected among normal choroid plexus epithe-

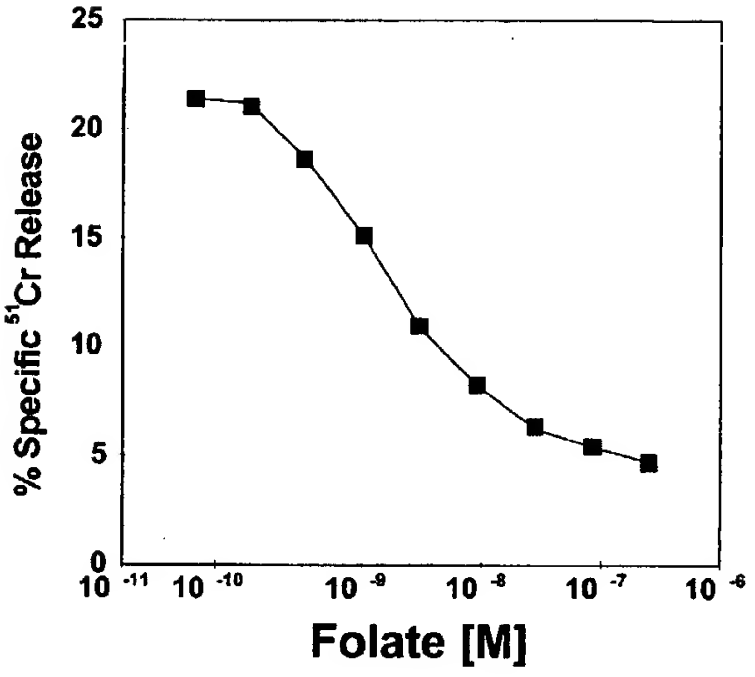


FIGURE 2 – Specific redirection of CTL clone 2C cytotoxic activity against CPT cells by 2C11/folate conjugate. To test the susceptibility of CPT cells to lysis by CTLs mediated through redirection by the 2C11/folate conjugate, ^{51}Cr-labeled CPT cells were incubated over a 4 hr period with CTL 2C, 150 ng/ml 2C11/folate conjugate and various concentrations of competing free folate. Results presented are the mean of triplicate samples.

lium, brain parenchyma or the ependymal lining of the brain ventricles (data not shown). Marginal apoptosis of CPT cells was observed in animals treated with activated splenocytes without 2C11/folate conjugate or saline alone (Fig. 4*e,f*). Further, apoptosis was not observed in CPT of animals treated with activated splenocytes and 2C11/folate conjugate 48 hr or longer after ICV transfer (data not shown).

2C11/folate conjugate retains T lymphocytes in CPT for periods of up to 1 week

A time course study revealed the duration that the 2C11/folate conjugate retained T cells in tumor tissue. SV11 mice were injected ICV with *in vivo*-activated splenocytes in the presence or absence of 2C11/folate conjugate. Subsequently, animals were perfused at various time points and brain sections stained for the presence of CD3. Figure 5 illustrates the mean density of T cells in 2C11/folate conjugate-treated animals over time compared with animals receiving T cells without 2C11/folate conjugate or with untreated SV11 animals. Corroborating results shown in Figure 4*b*, at 24 hr post-ICV delivery the density of T cells in CPT tissue of animals receiving no 2C11/folate conjugate at 24 hr was low, with an average of less than 25 T cells/mm^2. Similarly, the baseline presence of T cells within untreated SV11 animals was low, averaging 3 T cells/mm^2. In marked contrast, at 24 hr post-ICV delivery, animals receiving T cells with 2C11/folate conjugate had elevated levels of T cells among tumor tissue averaging over 600 T cells/mm^2 ($p < 0.01$ presence *vs.* absence of 2C11/folate conjugate) and in certain regions T-cell densities were over 1,500 T cells/mm^2. Further, elevated numbers of T cells can be found in

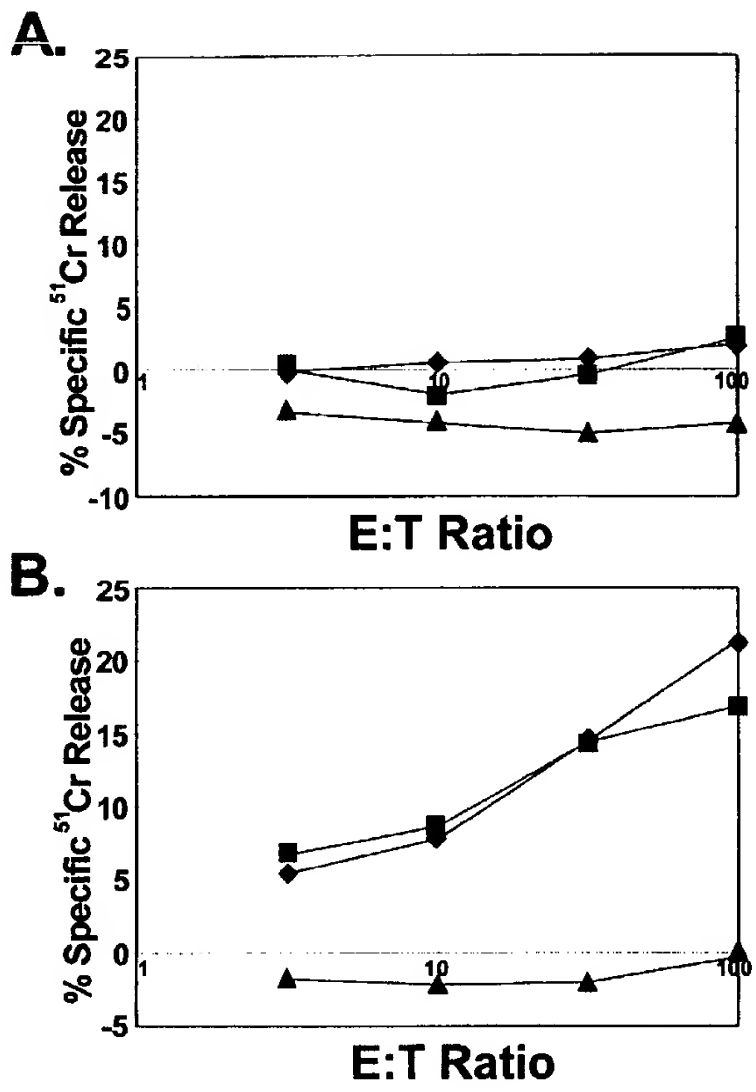
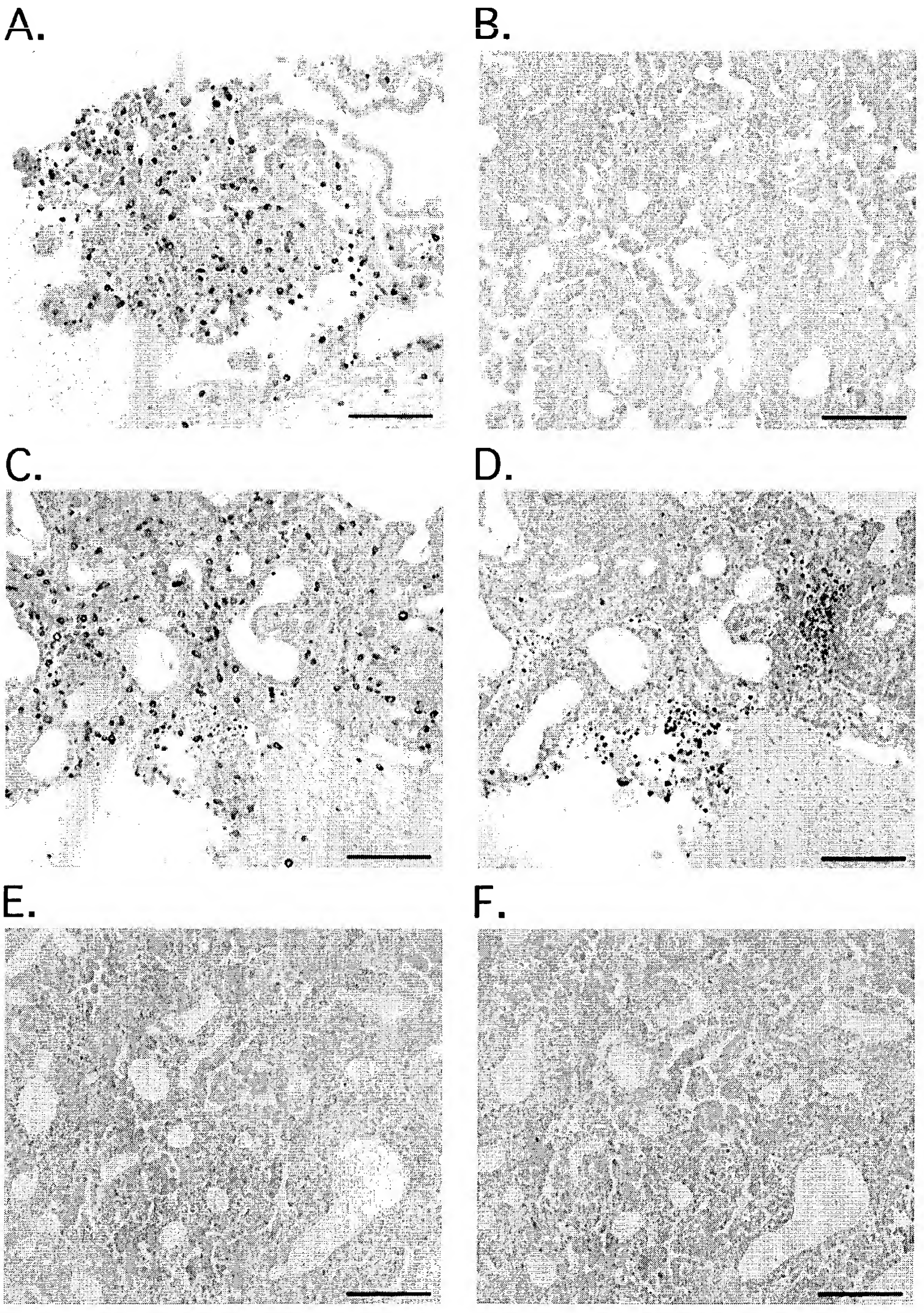


FIGURE 3 – Specific redirection of activated splenocytes to lyse ^{51}Cr-labeled CPT cells. The capacity for *in vivo*-activated effector CTLs among activated splenocytes to be redirected to lyse CPT cells by 2C11/folate conjugate was tested. Activated C57BL/6 (■), SV11 (♦) or unactivated SV11 (▲) splenocytes were incubated at various effector to target ratios for 4 hr in the absence *(a)* or presence *(b)* of 2C11/folate conjugate and CPT cells. Specific ^{51}Cr-release mediated by 2C11/folate conjugate redirection of CTL activity was inhibited by free folate (data not shown).

tumor tissue for up to 7 days compared to the no 2C11/folate conjugate 24 hr time point. The density of T cells in CPT 24 hr following delivery of activated splenocytes and unlabeled 2C11 antibody was similar to the density of delivering activated splenocytes alone (data not shown).

To control for the possibility that the T cells observed in the sections were not those adoptively transferred but immigrating endogenous cells, we analyzed the effects of injecting 2C11/folate conjugate or unlabeled 2C11 MAb alone into the lateral ventricles of SV11 animals. Injection of 2C11/folate conjugate ICV without splenocytes results in the immigration of a negligible density of T cells into CPT (data not shown). Even under circumstances where a preactivating dose of anti-CD3 antibody was administered systemically to SV11 animals 24 hr prior to ICV delivery of

FIGURE 4 – Retention of T cells in CPT and apoptosis. Animals were infused ICV with activated splenocytes in the presence *(a)* or absence *(b)* of 2C11/folate conjugate and killed 24 hr later. Tissue sections from multiple brain regions were stained for CD3. T cells are present throughout CPT tissue when 2C11/folate conjugate was administered *(a)*, but T cells have migrated out by 24 hr if 2C11/folate conjugate was not administered *(b)*. Relatively few T cells were observed in normal brain parenchyma. Adjacent sections from an animal treated with 2C11/folate and activated splenocytes killed 24 hr after adoptive transfer ICV were stained for CD3 *(c)* and apoptosis *(d)*. Again, 2C11/folate conjugate retained T cells specifically within CPT *(c)*. Cells staining for CD3 *(c)* are juxtaposed to apoptotic cells *(d)*. Apoptosis was not observed in normal brain parenchyma or choroid plexus. Animals not receiving 2C11/folate conjugate revealed few retained T cells in CPT at 24 hr *(e)* or apoptosis of tumor tissue on adjacent sections *(f)*. Scale bar for *a–f*: 100 μm.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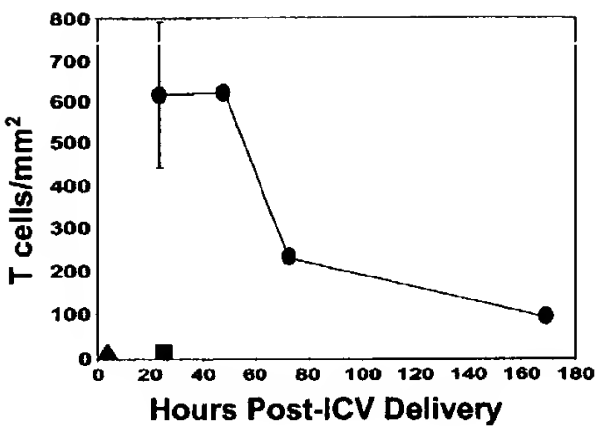

FIGURE 5 – T-cell retention in CPT following ICV delivery. Approximately 2×10^6 activated splenocytes were injected ICV in the presence (●) or absence (■) of 2C11/folate conjugate and compared with T-cell density in CPT of untreated SV11 animals (▲). SV11 animals were killed at various time points following injection, perfusion fixed and brains mounted for anti-CD3 immunohistochemistry. Total tumor area of lateral ventricle CPT of either treated or sham control animals was calculated from 3–5 brain sections of differing regions and the T cells quantified were associated with tumor. Vertical bars indicate SD. The number of animals used at each time point: 0 hr, n = 3; 24 hr, n = 4 for each; 48 hr, n = 1; 72 hr, n = 1; 168 hr, n = 1. Activated splenocytes with 2C11/folate differed from both untreated controls and activated splenocytes without conjugate, $p < 0.01$.

2C11/folate conjugate in attempts to promote CTL activation and surveillance, a negligible increase in density of T cells was observed infiltrating into CPT. Collectively, the observations indicate that the most probable origin of the T cells within sections of the CPT is that which is adoptively transferred.

Survival analysis of SV11 animals with a single adoptive transfer of activated splenocytes and 2C11/folate conjugate

SV11 mice received a single ICV injection of *in vivo*-activated splenocytes and 2C11/folate conjugate, splenocytes alone or PBS. Survival results indicated that administration of splenocytes and 2C11/folate conjugate in a single bolus ICV did not confer a statistically significant therapeutic advantage over administering T cells alone ($p > 0.05$) or sham controls ($p > 0.05$). While animal longevity was not extended, fewer animals appear to become moribund at younger ages (85–100 days) when treated with T cells (with or without 2C11/folate conjugate) compared to sham controls.

Comparison of in vivo- vs. in vitro-*activated splenocyte effectors*

Flow cytometric analysis of *in vivo*-activated splenocytes harvested for ICV delivery demonstrates that the effector population of activated $CD8^+$ cells accounted for only 4–9% of total splenocytes compared with an average of 15–20% in a resting spleen (Fig. 6*a,b*). CD69, an early marker indicative of T-lymphocyte activation (Allison *et al.*, 1995), was present on approximately 70–80% of $CD4^+$ or $CD8^+$ cells in animals receiving 2C11, but on less than 2% of T lymphocytes in animals not receiving 2C11. Thus, injection of 3.5×10^6 splenocytes resulted in fewer than 3.5×10^5 $CD8^+$ cytotoxic T cells actually delivered and fewer still likely capable of cytolysis. Due to the low proportion of $CD8^+$ cells rendered with *in vivo* activation, splenocytes were activated *in vitro* with a combination of anti-CD3 (2C11) and anti-CD28 (37.51) antibodies to expand the proportion of CTL effectors in the overall cell population. Splenocytes were incubated with 100 ng/ml 2C11 antibody and various doses of 37.51 to determine the optimal level for producing active CTL effectors. Activation of splenic T cells with CD28 costimulation after 72 hr consistently expanded the $CD8^+$ population to 60–70% of total cells regardless of the 37.51 dose tested (approximately 10 times the percentage of $CD8^+$ cells found among *in vivo*-activated splenocytes). Representative flow cytometric data for the *in vitro*-activated splenocytes for $CD8^+$ cells are shown in Figure 6*c*. CD69 expression is not observed on the predominance of T lymphocytes following *in vitro* activation most likely as a result of the relatively transient expression of this marker.

We tested the relative effectiveness of *in vitro*- and *in vivo*-activated splenocytes at lysing a FR-expressing target mediated by 2C11/folate redirection. Similar to *in vivo*-activated CTLs, *in vitro*-activated CTLs demonstrate the capacity to lyse the FR-expressing cell line F2-MTX^{r}A in the presence of 2C11/folate conjugate (Fig. 7*a,b*). Comparison of the lytic potential of these 2 effector populations revealed that the ER_{50} (effector to target ratio giving half-maximal lysis) is approximately 6- to 10-fold lower for *in vitro*-activated effectors (10 times fewer *in vitro*-activated splenocytes compared to *in vivo*-activated splenocytes). Given that the percentage of $CD8^+$ cells among *in vitro*-activated splenocytes is approximately 10-fold greater than that among the *in vivo*-activated splenocytes, the resulting 10-fold difference in the ER_{50} likely is a function of CTL number and not differences in CTL cytotoxic potency.

Survival analysis of SV11 animals treated with multiple adoptive transfers of in vitro-*activated polyclonal effectors and 2C11/folate conjugate through indwelling cannulas*

Two groups of mice receiving *in vitro*-activated cells every 3 or 5 days, either with 2C11/folate conjugate (n = 18) or without (n = 11), were compared to a group of animals receiving saline (n = 6). Survival analysis demonstrated that cotreatment of CPT with activated splenocyte effector cells and 2C11/folate conjugate improved survival over administration of T cells alone ($p = 0.001$) or saline ($p = 0.003$) (Fig. 8). Mean time to morbidity of animals treated with T cells alone was 98 days (SD 1, median 98) while animals receiving 2C11/folate conjugate survived an average of 107 days (SD 2, median 107) indicating that the presence of 2C11/folate conjugate conferred a therapeutic advantage. Mean time to morbidity of saline controls was 99 days (SD 1, median 98).

DISCUSSION

The aim of our study was to determine whether a bispecific ligand-antibody conjugate could produce a significant improvement in survival of animals with an endogenously arising solid brain tumor, when delivered directly to the tumor. The first objective was to determine whether CPT cells would be susceptible to bispecific ligand-antibody conjugate-mediated cytolysis by polyclonal effector cells derived from SV11 mice or congeneic C57BL/6 mice. Some types of brain tumors secrete substances that inhibit the activation of T cells, *e.g.*, TGF-β. The possibility exists that the CPT in the SV11 animal provides a locally immunosuppressive microenvironment. Unactivated polyclonal splenocytes from SV11 animals showed no detectable lytic activity against CPT, suggesting that either the SV11 animal is tolerant to the presence of tumor or that the tumor may be locally or systemically immunosuppressing the mice. However, anti-CD3-activated polyclonal effectors from SV11 or C57BL/6 mice could be induced to lyse CPT in the presence of 2C11/folate conjugate, demonstrating that if there is systemic immunosuppression, it can be overcome. Further, these same activated polyclonal effectors from SV11 mice did not lyse CPT in the absence of 2C11/folate conjugate, suggesting that SV11 mice do not harbor expanded antitumor clones among splenocytes. Folate competition of CTL-mediated lysis demonstrated that the 2C11/folate conjugate was responsible for the cytotoxic interaction. In addition, incubating target cells with active CTL and unlabeled 2C11 antibody did not lead to cytolysis of target cell lines *in vitro* (data not shown).

When administered *in vivo*, the 2C11/folate conjugate was capable of retaining T lymphocytes within CPT tissue, whereas these same effectors quickly cleared from the brain in the absence of 2C11/folate conjugate. These data suggest that the 2C11/folate

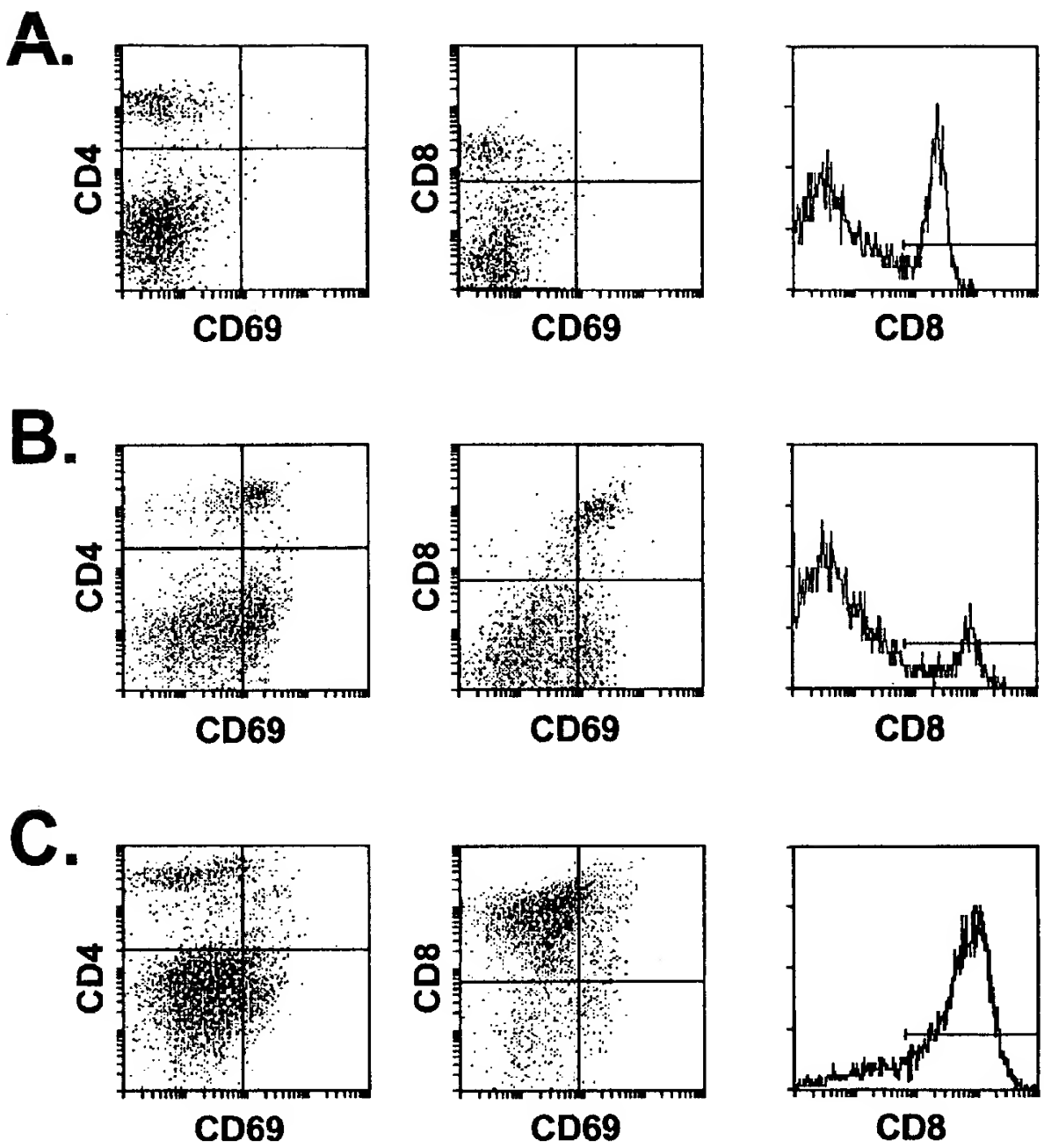

FIGURE 6 – Flow cytometric analysis of activated splenocytes. Resting *(a)* or *in vivo*-activated *(b)* splenocytes were labeled with a combination of labeled anti-CD4, CD8 and CD69 antibodies. Resting splenocytes have $CD4^+$ and $CD8^+$ populations of approximately 25–35% and 15–20%, respectively, and low expression of the early activation marker CD69. Twenty-four hours following a 10 μg injection of 2C11, the majority of $CD4^+$ and $CD8^+$ cells are positive for CD69, indicating an activated state, although the percentage of $CD8^+$ cells has markedly decreased among splenocytes (<8%). Splenocytes activated *in vitro (c)* were similarly labeled with a combination of anti-CD4, CD8 and CD69 antibodies following a 72 hr incubation period with 100 ng/ml 2C11 and 0.5 mg/ml 37.51. The percentage of $CD8^+$ cells has expanded (68%). The early activation marker CD69 largely is not present on these cells, indicating marker turnover rather than an unactivated state.

conjugate is capable of contributing to the prevention of the normal migratory tendency of activated T lymphocytes. The data also indicate that the 2C11/folate conjugate is capable of retaining T lymphocytes in CPT tissue for periods of up to 1 week at higher than background densities.

The first treatment protocol involved adoptive delivery of a single injection of activated splenocytes and 2C11/folate conjugate. The animals were treated at 85–90 days of age, a point at which the CPTs typically have progressed to grade III and IV neoplasms. The survival of SV11 animals treated in this fashion did not significantly differ from that of animals treated with activated splenocytes alone or saline-injected animals. Considering the relative density of T cells adhering to CPTs in the time course analysis, calculations indicate that perhaps 1 T cell is present for every 4–10 CPT cells at 24–48 hr post-ICV delivery. The low proportion of $CD8^+$ cells among *in vivo*-activated splenocytes and the relatively short duration of peak retention of T-cell density suggested the need for enriching the proportion of $CD8^+$ CTLs among the splenocyte pool as well as devising a means to deliver multiple injections of CTL and 2C11/folate conjugate.

Activating splenocytes *in vitro* greatly expanded the CTL effector population. Although CTLs activated in this fashion are not more potent against CPT cells than the *in vivo*-activated counterparts, more CTLs could be delivered per injection. Delivering multiple infusions of splenocytes and 2C11/folate conjugate through indwelling cannulas increased SV11 animal survival. Activating splenocytes *in vitro* results in an effector CTL population that is precoated with unlabeled 2C11 antibody (flow cytometric results, data not shown). Repeated delivery of splenocytes alone into the lateral brain ventricles in these experiments actually includes delivery of 2C11 antibody. Because splenocytes precoated with 2C11 did not confer a therapeutic advantage over animals treated with saline, the 2C11/folate conjugate was responsible for conferring the beneficial therapeutic effect.

To further characterize CTL action against CPT *in vivo* and possible side effects to choroid plexus epithelium or healthy brain

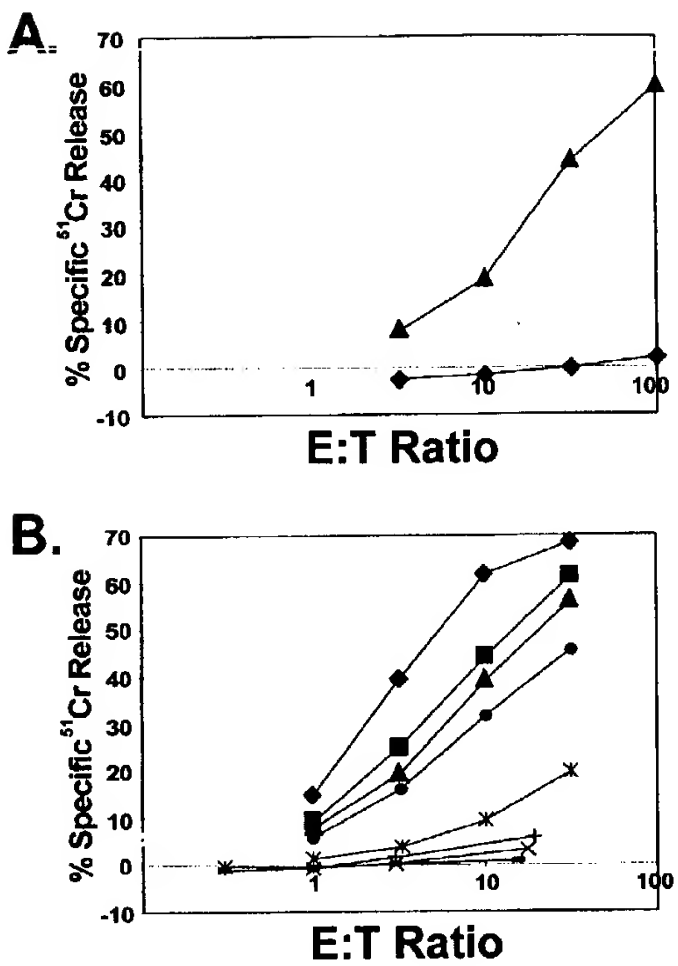

FIGURE 7 – Comparison of *in vivo*- and *in vitro*-activated splenocyte retargeting. The FR^+ tumor target line F^2-MTX^rA and various effector to target (E:T) ratios of each effector population were incubated with 150 ng/ml 2C11/folate. *In vivo*-activated effectors *(a)* were tested either in the presence (▲) or absence (♦) of 2C11/folate conjugate at E:T ratios of 100:1, 30:1, 10:1 and 3:1. Splenocytes activated *in vitro (b)* were stimulated with 100 ng/ml 2C11 and various concentrations of 37.51:5 μg/ml (♦), 1 μg/ml (■), 0.2 μg/ml (▲), 0.04 μg/ml (●) and 0.008 μg/ml (*). Splenocytes were also incubated with 100 ng/ml 2C11 alone (+), 5 μg/ml 37.51 alone (×) or media alone (—). E:T ratios tested for all *in vitro*-activated splenocytes were 30:1, 10:1, 3:1 and 1:1. Half-maximal lysis (ER_{50}) was achieved at an E:T of approximately 30:1 by *in vivo*-activated splenocytes, but an ER_{50} of less than 5:1 for *in vitro*-activated splenocytes.

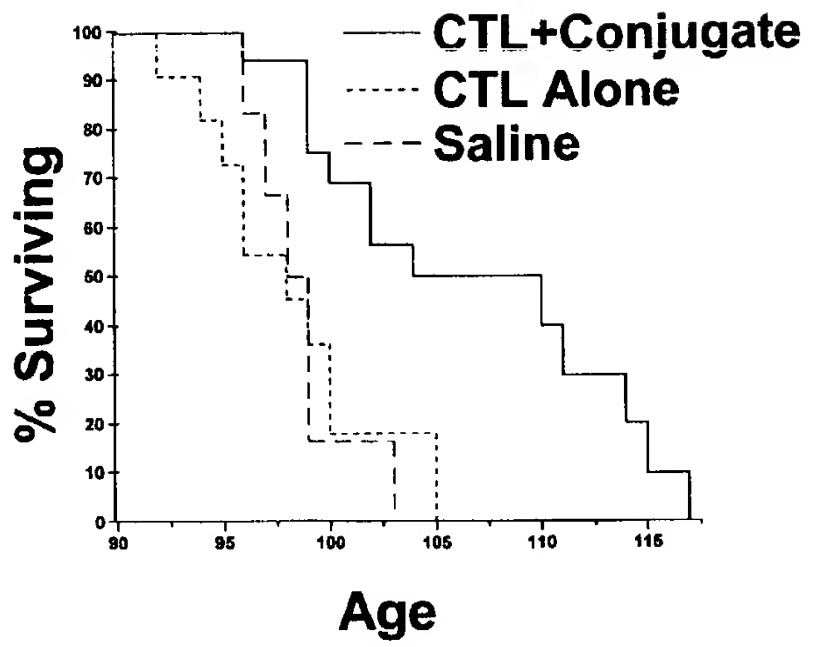

FIGURE 8 – Survival of cannulated SV11 mice treated ICV with multiple injections of *in vivo*-activated splenocytes and 2C11/folate conjugate. SV11 animals were injected ICV at approximately 85 days of age and every 3–5 days thereafter until morbidity. Animals received saline (——) (n = 6), preactivated splenocytes (---) (n = 11) or preactivated splenocytes and 1 μg 2C11/folate conjugate (—) (n = 18). Animals were assessed daily for alteration in body weight and vestibular complications. A 25% decrease in pretreatment body weight or inability to right self served as criterion for morbidity. Animals treated with 2C11/folate conjugate and activated splenocytes lived significantly longer than animals treated with saline ($p = 0.003$) or activated splenocytes without conjugate ($p = 0.001$).

parenchyma, we performed apoptotic assays of tissue sections adjacent to those stained for CD3. Untreated SV11 animals commonly have evidence of a slight amount of background apoptosis in CPT tissue. Apoptosis of CPT cells of SV11 animals treated with saline or activated splenocytes alone was not appreciably different from untreated SV11 animals. We observed a greater extent of apoptotic staining in CPT of SV11 animals receiving 2C11/folate conjugate and activated splenocytes. Adjacent slide sections stained for apoptosis or the CD3 antigen showed apoptotic cells to be juxtaposed to cells staining for CD3, suggesting that the 2C11/folate conjugate may be redirecting CTL action against CPT cells. Apoptosis of non-tumor tissue was not observed in any of the treatment group animals nor in normal C57 mice treated under similar conditions.

T cells delivered into the lateral ventricles with 2C11/folate conjugate were found to penetrate tumors located in all regions of the lateral or third ventricles, but IVth ventricle tumors were typically devoid of T-cell presence, suggesting that T cells are not penetrating the cerebral aqueduct following ICV delivery. A number of SV11 animals receiving CTL and 2C11/folate conjugate had small or negligible tumor loads in the lateral ventricles at later survival times, compared with the typically large lateral ventricle tumors of animals not receiving 2C11/folate conjugate, and large IVth ventricle tumors.

A potential cause for concern regarding the ICV treatment regime is that normal choroid plexus epithelium expresses the high-affinity folate receptor as do CPTs. Normal choroid plexus has a polarized distribution of FR concentrated on the apical surface facing the ventricular lumen directly exposed to the injected T cells and 2C11/folate conjugate (Patrick *et al.*, 1997). In numerous cases, T cells were found associated with normal choroid plexus, although predominately in the presence of 2C11/folate conjugate. While the choroid plexus was sometimes altered in morphology compared with that of untreated animals, we were unable to detect damage to normal choroid plexus in treated mice, either by gross morphology or presence of apoptotic cells. We do not know why epithelial cells appeared resistant to T-cell-mediated lysis. Similarly, C57 mice treated with activated T cells and 2C11/folate conjugate did not show any signs of short-term (12–24 hr) or long-term (up to 1 year) toxicity to normal choroid plexus.

Given the transgenic nature of the CPT, we did not expect any of the treatment regimes to produce a complete cure. All cells of the choroid plexus epithelium may serve as potential origins of tumorigenesis. SV11 animals commonly have multiple CPT foci in the lateral and IVth ventricles at morbidity. A more realistic goal in this model is comparing increases in mean time to morbidity rather than expecting long periods of disease-free remission.

While treatment of SV11 animals with multiple injections of splenocytes and 2C11/folate conjugate improved animal survival, there is clearly room for improvement. An important issue to address is the length of time retained CTLs remain active and capable of cytolysis within the CPT. While T cells were observed adhering to CPT for periods beyond 1 week post-ICV delivery, the activational status of these cells is unknown. Considering that primary T-cell activation is a transient process for an individual T cell, CTLs are unlikely to remain active for extended periods of

time (Speiser *et al.*, 1997). In addition, activation of CTL with 2C11 leads to a down-regulation of CD3 that may have reduced the potential efficacy of the bispecific ligand-antibody conjugate strategy for treatment of CPTs. As a potential means of promoting greater longevity of CTL activation, the application of additional bispecific ligand-antibody conjugates targeting costimulatory molecules (*e.g.*, folate/anti-CD28, folate/anti-LFA-1) may simultaneously enhance primary activation or permit reactivation of adoptively transferred cells. An additional strategy may be to deliver blocking anti-CTLA-4 monovalent antibody fragments to reduce possible CTL down-regulation (Allison *et al.*, 1995).

Targeting sufficient numbers of activated effectors to tumor has been a primary concern for most immunotherapeutic strategies. The high affinity of the FR for folate and the apparent capability of the 2C11/folate conjugate to keep T cells retained in tumor for periods of up to 1 week suggest that attaching a high-affinity ligand to antieffector cell antibodies may be an effective means of targeting and retaining effector cells in tumor regions. The incorporation of bispecific ligand-antibody conjugates selectively stimulating costimulatory molecules on effector cells may promote sustained reactions to tumor, and perhaps the recruitment of endogenous effectors.

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